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CLONING AND EXPRESSION OF HTLV-III DNA

Description

Technical Fields

This invention is in the fields of biology and virology and in particular relates to human T cell leukemia virus - type III (HTLV-III).

Background Art

The term human T cell leukemia-lymphoma virus (HTLV) refers to a unique family of T cell tropic retroviruses. Such viruses play an important role in the pathogenesis of certain T cell neoplasms. There are presently three known types of HTLVs. One subgroup of the family, HTLV-type I (HTLV-I) is linked to the cause of adult T-cell leukemia-lymphoma (ATLL) that occurs in certain regions of Japan, the Caribbean and Africa. HTLV-type II (HTLV-II) has been isolated from a patient with a T-cell variant of hairy cell leukemia. M. Popovic et al., Detection, Isolation, and Continuous Production of Cytopathic Retroviruses (HTLV-III) from

Patients with AIDS and Pre-AIDS	Science	224:497-
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HTLV-type III (HTLV-III) has been isolated from many patients with acquired immune deficiency syndrome (AIDS). It refers to prototype virus isolated from AIDS patients. Groups reported to be at greatest risk for AIDS include homosexual or bisexual males; intravenous drug users and Haitian immigrants to the United States.

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hemophiliacs who receive blood products pooled from donors and recipients of multiple blood transfusions are also at risk. Clinical manifestations include severe, unexplained immune deficiency which generally involves a depletion of helper T lymphocytes. These may be accompanied by malignancies and infections. The mortality rate for those with AIDS is high. A less severe form of AIDS also exists, in which there may be lymphadenopathy and depressed helper T cell counts; there is not, however, the devastating illness characteristic of full-blown AIDS. There are many individuals, who are classified as having early AIDS (pre-AIDS), who exhibit these signs. It is not now possible to predict who among them will develop the more serious symptoms.

Much of the evidence implicates HTLV-III as the etiological agent of the infectious AIDS. First, there is consistent epidemiology; greater than 95% of the patients with AIDS have antibodies specific for HTLV-III. Second, there has been reproducible identification and isolation of virus in this disease; more than 100 variants of HTLV-III have been isolated from AIDS patients. Third, there has been transmission of the disease to normal healthy individuals who received blood transfusions from infected blood donors.

HTLV-III has been shown to share several properties with HTLV-I and HTLV-II but also to be morphologically, biologically and antigenically distinguishable. R.C. Gallo et al., Frequent Detection and Isolation of Cytopathic Retroviruses (HTLV-III) from Patients with AIDS and At Risk for AIDS. Science, 224:500-503. (1984). For example,

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HTLV-III has been shown to be antigenically related to HTLV-I and HTLV-II by demonstrating cross-reactivity with antibodies to HTLV-I and HTLV-II core proteins, P24 and P19, and envelope antigens and by nucleic acid cross-hybridization studies with cloned HTLV-I and HTLV-II DNAs. However, unlike HTLV-I and HTLV-II, it lacked the ability to infect and transform T cells from normal umbilical cord blood and bone marrow in vitro, and has the cytopathic effect on infected cells only.

Like the RNA genome of other retroviruses, the RNA genome of HTLV-III contains three genes which encode viral proteins: 1) the gag gene, which encodes the internal structural (nucleocapsid or core) proteins; 2) the pol gene, which encodes the RNA-directed DNA polymerase (reverse transcriptase); and 3) the env gene, which encodes the envelope glycoproteins of the virion. In addition, the HTLV-III genome contains a region designated Px, located between the env gene and the 3' LTR, which appears to be involved in functional killing of the virus.

At this time, AIDS is still difficult to diagnose before the onset of clinical manifestations and impossible to treat or even prevent.

Summary of the Invention

This invention is based upon applicant's cloning of HTLV-III DNA in recombinant/vector host systems capable of expressing immunoreactive HTLV-III polypeptides. In one embodiment, an immunoreactive protein coded for by an env gene sequence of HTLV-III has been produced by these recombinant

DNA methods. This polypeptide is immunoreactive with sera of patients having acquired immunodeficiency syndrome or antibodies to HTLV-III. The polypeptide expressed has been isolated.

In another embodiment of the invention, immunoreactive polypeptides produced by the recombinant DNA methods are employed in the production of antibodies, including monoclonal antibodies, reactive with the polypeptides. Such antibodies form the basis for immunoassay and diagnostic techniques for detecting HTLV-III, particularly in body fluids such as blood, saliva, urine, etc.

In another embodiment of the invention, DNA probes are formed from DNA sequences coding for portions of the HTLV-III genome. Such DNA probes can also be employed in detecting the presence of HTLV-III in blood or other fluids.

Diagnostic kits including immunoreactive polypeptides, DNA probes, etc. can also be produced to include any of the products of this invention.

Brief Description of the Figures

Figure 1 is a representation of HTLV-III DNA. Figure 1a shows sites at which the genome is cut by the restriction enzyme SstI and Figure 1b shows the fragments of HTLV-III genome produced through the action of restriction enzymes Kpn, EcoRI and Hind III.

Figure 2 is a representation of HTLV-III DNA and the location of restriction enzyme sites in the genome.

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Figure 3 shows nucleotide sequences for HTLV-III DNA which encompasses the env region.

Figure 4 is an immunoblot showing the position on an SDS polyacrylamide gel of HTLV-III env-Beta-galactosidase fusion proteins.

Best Mode of Carrying Out the Invention

The envelope glycoprotein is the major antigen recognized by the antiserum of AIDS patients. In this respect, HTLV resembles other retroviruses, for which the envelope glycoprotein is typically the most antigenic viral polypeptide. In addition, the neutralizing antibodies are generally directed toward the envelope glycoprotein of the retrovirus. Serum samples from 88 percent to 100 percent of those with AIDS have been shown to have antibodies reactive with antigens of HTLV-III; the major immune reactivity was directed against p41, the presumed envelope antigen of HTLV-III. Antibodies to core proteins have also been demonstrated in serum of AIDS patients, but are evidently not as effective an indicator of infection as is the presence of antibodies to envelope antigen.

The p41 antigen of HTLV-III has been difficult to characterize because the viral envelope is partially destroyed during the process of virus inactivation and purification. The present invention responds to the great need to characterize the antigenic component of the HTLV-III virus--and thus provide screening, diagnostic and preventive products and methods--in several ways.

First, the present invention relates to the isolation of genes of HTLV-III which encode

immunoreactive polypeptides; identification of the nucleotide sequence of these genes; introduction of DNA sequences specific to these viral DNA sequences into appropriate vectors to produce viral RNA and the formation of DNA probes. These probes are comprised of sequences specific to HTLV-III DNA and are useful, for example, for detecting the same HTLV-III DNA sequences in body fluids (e.g., blood).

Second, the present invention relates to HTLV-III polypeptides which are produced by translation of the recombinant DNA sequences encoding HTLV-III proteins. Polypeptides which are so produced and which are immunoreactive with serum from AIDS patients are referred to as recombinant DNA-produced immunoreactive HTLV-III polypeptides. They include, but are not limited to, antigenic HTLV-III core and envelope polypeptides which are produced by translation of the recombinant DNA sequences specific to the gag and the env DNA sequences encoding HTLV-III core proteins and envelope glycoproteins, respectively. They also include the polypeptides which are produced by translation of the recombinant DNA sequences specific to the Px genes of HTLV-III. The polypeptides may be used as vaccines for the prevention of AIDS. The methods of producing the polypeptides are also a subject of this invention, as are diagnostic methods based on these polypeptides.

Third, the present invention also relates to antibodies against the immunoreactive HTLV-III polypeptides which are the subject of this invention. These antibodies are the basis for assays

relating to the diagnosis of AIDS or the presence of HTLV-III in body fluids.

In one embodiment of this invention, genetic engineering methods are used to isolate DNA sequences of HTLV-III which encode immunoreactive HTLV-III polypeptides, such as the core protein and the envelope glycoprotein, and to identify the nucleotides which comprise those sequences. The proviral genes integrated into host cell DNA are molecularly cloned and the nucleotide sequences of the cloned provirus is determined.

An E. coli expression library of HTLV-III DNA is constructed; in this library are vectors harboring HTLV-III DNA sequences. The HTLV-III genome is cloned and cuts are then made in the cloned HTLV-III genome with restriction enzymes to produce DNA fragments. (Figures 1 and 2) HTLV-III DNA fragments of approximately 200-500bp are isolated from agarose gel, end repaired with T₄ polymerase and ligated to linker DNA. The linker ligated DNA is then treated with a restriction enzyme, purified from agarose gel and cloned in an expression vector. Examples of the expression vectors used are: OmpA, pIN (A,B and C), lambda pL, T7, lac, Trp, ORF and lambda gt11. In addition, mammalian cell vectors such as pSV28pt, pSV2neo, pSVdhfr and VPV vectors, and yeast vectors, such as GALI and GAL10, may be used.

The bacterial vectors contain the lac coding sequences, into which HTLV-III DNA can be inserted for the generation of B-galactosidase fusion protein. The hybrid molecules are then introduced into bacteria (e.g., E.coli); those cells which take up a

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vector containing HTLV-III DNA are said to be transformed. The bacteria are plated on top of MacConkey agar plates in order to verify the phenotype of clone. If functional B-galactosidase is being produced, the colony will appear red.

Bacterial colonies are also screened with HTLV-III DNA probes containing the DNA regions of interest (e.g., HTLV-III gag and env DNA sequences). This results in identification of those clones containing the insert. Clones which are positive when screened with the DNA probe and positive on the MacConkey agar plates are isolated.

This identification of cells harboring the HTLV-III DNA sequences makes it possible to produce HTLV-III polypeptides which are immunoreactive with HTLV-III specific antibody. The cells from the selected colonies are grown in culture under conditions conducive to allowing the expression of the hybrid protein. The culture is spun down and the resulting cell pellet broken. The total cellular protein is analysed by being run on an SDS polyacrylamide gel. The fusion proteins are identified at a position on the gel which contains no other protein. (Figure 2) Western blot analyses are also carried out on the clones which screened positive. Such analyses are carried out using serum from AIDS patients, with the result that it is possible to identify those clones expressing HTLV-III env-B-galactosidase fusion proteins (antigens) that cross-react with the HTLV-III specific antibody.

In another embodiment of this invention, lambda ₁₀ clones harboring HTLV-III DNA are cloned

from the replicated form of the virus. As the retrovirus is replicating, double stranded DNA is being produced. Cuts are made in the cloned HTLV-III DNA with the restriction enzyme SstI. (Figure 1a) Because there are two SstI recognition sites within the LTR of HTLV-III DNA, one LTR region is not present in the cloned DNA sequence removed from the lambda₁₀ vector. As a result, a small (approximately 200 bp) fragment of the HTLV-III DNA is missing.

The resulting DNA is linearized and fragments are produced by digesting the linearized genomic DNA spanning the env gene region with restriction enzymes. For example, fragments are produced using Kpn or EcoRI plus HindIII, as shown in Figure 1b. The resulting 2.3kb KpnI-KpnI fragments; 1.0kbEcoRI-EcoRI fragments and 2.4Kb EcoRI-HindIII fragments are isolated by gel electrophoresis and electro-elution. These fragments are randomly sheared to produce fragments. The fragments thus produced are purified from agarose gel and DNA fragments between about 200-500 bp are eluted.

The eluted 200-500bp DNA fragments are end filled through the use of E. coli T₄ polymerase and blunt end ligated into an open reading frame expression (ORF) vector, such as pMR100. This ligation may occur at the SmaI site of the pMR100 vector, which contains two promoter regions, hybrid coding sequences of lambdaCI gene and lacI-LacZ gene fusion sequence. In the vector, these are out of frame sequences; as a result, the vector is nonproductive. The HTLV-III DNA is inserted into the vector; the correct DNA fragments will correct the reading

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frame, with the result that CI-HTLV-III-B-galactosidase fusion proteins are produced. The expression of the hybrid is under the control of the lac promoter.

Based on the sequence of pMR100, it appears that if a DNA fragment insert cloned into the SmaI site is to generate a proper open reading frame between the lambdaCI gene fragment and the lac-7 fragment, the inserted DNA must not contain any stop codons in the reading frame set by the frame of the lambdaCI gene.

The hybrid molecules are then introduced into E. coli. The bacteria are plated on MacConkey agar plates to verify the phenotype of the clone. If functional B-galactosidase is being produced, the colony will appear red. The colonies are also screened with HTLV-III DNA probes, for the purpose of identifying those clones containing the insert. Clones which are positive when screened with the DNA probe and positive on the MacConkey agar plates are isolated.

The cells from the selected colonies are grown in culture. The culture is spun down and the cell pellet broken. Total cellular protein is analysed by being run on an SDS polyacrylamide gel. The fusion proteins are identified at a position on the gel which contains no other protein. (Figure 4)

Western blot analyses are also carried out on the clones which screened positive. Sera from AIDS patients are used, thus making it possible to identify those clones which express the HTLV-III-env-B-galactosidase fusion proteins (antigens) that cross-react with the HTLV-III specific antibody.

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1000 clones were screened by this method; 6 were positive.

Because of the nature of the pMR100 cloning vehicle, a productive DNA insert should also be expressed as a part of a larger fusion polypeptide. HTLV-III env gene containing recombinant clones was identified by colony hybridization. The production of larger fusion polypeptides bearing functional B-galactosidase activity was verified by phenotype identification on MacConkey agar plates; by B-galactosidase enzymatic assays and by analysis on 7.5% SDS-polyacrylamide gels. Immunoreactivity of the larger protein with antibody to HTLV-III was assessed by western blot analysis using serum from AIDS patients. These large fusion proteins also reacted with anti-B-galactosidase and anti-CI antiserum. This finding is consistent with the hypothesis that they are proteins of CI-HTLV-III-lacIZ.

The open reading frame insert fragment of HTLV-III is further analyzed by DNA sequencing analysis. Because one of the two BamHI sites flanking the SmaI cloning site in pMR100 is destroyed in the cloning step, positive clones are digested with restriction enzymes HindIII and *clai*I to liberate the inserted HTLV-III DNA fragment. The HTLV-III ORF inserts are isolated from the fusion recombinant and cloned into M13 sequencing cloning vector mp18 and mp19 digested with HindIII and *Acc*I. DNA sequences of the positive ORF clones are then determined.

In another embodiment of this invention, fragments of HTLV-III DNA of approximately 200-500

bps are isolated from agarose gel, end repaired with T_4 polymerase and ligated to EcoRI linker. The EcoRI linker ligated DNA is then treated with EcoRI purified from 1% agarose gel and cloned in an expression vector, gtl1. This vector contains lac Z gene coding sequences into which the foreign DNA can be inserted for the generation of B-galactosidase fusion protein. The expression of the hybrid gene is under the control of lac repressor. The lac repressor gene, lac I, is carried on a separate plasmid pMC9 in the host cell, E. coli Y1090. AIDS patient serum was used to probe the gtl1 library of HTLV-III genome DNA containing 1.5×10^4 recombinant phage. In a screen of 5000 recombinants, 100 independent clones that produced strong signals were isolated. The positive recombinant DNA clones were further characterized for their specific gene expression. Rabbit hyperimmune serum against P24 was also used to identify the gag gene specific clones. Nick-translated DNA probes of specific HTLV-III gene, specifically the gag gene, env gene and Px gene were used to group the positive immunoreactive clones into specific gene region.

Recombinant clones that produced strong signals with AIDS serum and contain insert DNA spanning the HTLV-III env gene region were examined in detail by mapping their insert with restriction enzymes and DNA sequencing analysis.

Another embodiment of this invention relates to the formation of RNA and RNA probes specific to the HTLV-III DNA of this invention. DNA sequences which are an entire gene or segment of a gene from HTLV-III are inserted into a vector, such as a T7 vector.

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In this embodiment, the vector has the Tceu promoter from the T cell gene 10 promoter and eleven amino acids from the T cell gene 10 protein.

The vectors are then used to transform cells, such as E. coli. The T7 vector makes use of the T7 polymerase, which catalyzes RNA formation and recognizes only T7 promoter, which is the site where RNA polymerase binds for the initiation of transcription. This vector does not, however, recognize E. coli promoter. As a result, if HTLV-III DNA sequences are inserted after the promoter and polymerase genes of the T7 vector, which recognizes them to the exclusion of other signals, and a terminator is placed immediately after the HTLV-III DNA sequences, the T7 vector will direct manufacture RNA complementary to the HTLV-III DNA insert.

Monoclonal antibodies reactive with HTLV-III envelope polypeptide are produced by antibody-producing cell lines. The antibody-producing cell lines may be hybridoma cell lines commonly known as hybridomas. The hybrid cells are formed from the fusion of cells which produce antibody to HTLV-III envelope polypeptide and an immortalizing cell line, that is, a cell line which imparts long term tissue culture stability on the hybrid cell. In the formation of the hybrid cell lines, the first fusion partner - the antibody-producing cell - may be a spleen cell of an animal immunized against HTLV-III envelope polypeptide. Alternatively, the antibody-producing cell may be an anti-HTLV-III envelope polypeptide lymphocyte obtained from the spleen, peripheral blood, lymph nodes or other tissue. The

second fusion partner - the immortal cell - may be a lymphoblastoid cell or a plasmacytoma cell such as a myeloma cell, itself an antibody-producing cell but also malignant.

Murine hybridomas which produce monoclonal antibodies against HTLV-III envelope polypeptide are formed by the fusion of mouse myeloma cells and spleen cells from mice immunized against the polypeptide. To immunize the mice, a variety of different immunization protocols may be followed. For instance mice may receive primary and boosting immunizations of the purified polypeptide. The fusions are accomplished by standard procedures. Kohler and Milstein, (1975) Nature (London) 256, 495-497; Kennet, R., (1980) in Monoclonal Antibodies (Kennet et al., Eds. pp. 365-367, Plenum Press, NY).

The hybridomas are then screened for production of antibody reactive with envelope polypeptide.

Another way of forming the antibody-producing cell line is by transformation of antibody-producing cells. For example, a B lymphocyte obtained from an animal immunized against HTLV-III envelope polypeptide may be infected and transformed with a virus such as the Epstein-Barr virus in the case of human B lymphocytes to give an immortal antibody-producing cell. See, e.g., Kozbor and Rodor (1983) Immunology Today 4(3), 72-79. Alternatively, the B lymphocyte may be transformed by a transforming gene or transforming gene product.

The monoclonal antibodies against HTLV-III envelope polypeptide are produced in large quantities by injecting antibody-producing hybridomas into the peritoneal cavity of mice and, after an

appropriate time, harvesting the ascites fluid which contains very high titer of homogenous antibody and isolating the monoclonal antibodies therefrom. Xenogeneic hybridomas should be injected into irradiated or athymic nude mice. Alternatively, the antibodies may be produced by culturing cells which produce HTLV-III envelope polypeptide in vitro and isolating secreted monoclonal antibodies from the cell culture medium.

This invention will now be further illustrated by the following examples. They are not intended to be limiting in any way.

EXAMPLE 1

PREPARATION OF SONICATED DNA FRAGMENTS

10 ug of gel purified HTLV-III restriction fragments were sonicated to fragment size on average of 500 bps. After sonication, the DNA was passed through a DEAE-cellulose column in 0.1XTBE in order to reduce the volume. The DEAE-bound DNA was washed with 5 ml of 0.2 M NaCl-TE (2 M NaCl, 10 mM Tris HCl pH 7.5, 1 mM EDTA) and then eluted with 1 M NaCl-TE, and ethanol precipitated. The size range of the sonicated DNA was then determined on 1.2% agarose gel. DNA fragments of desired length (200-500 bps) was eluted from the gel. T4 DNA polymerase was used to fill in and/or trim the single strand DNA termini generated by the sonication procedure. DNA fragments were incubated with T4 polymerase in the absence of added nucleotides for five minutes at 37°C to remove nucleotides from 3' end and then all 4 nucleotide precursors were added to a final

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concentration of 100 uM and the reaction mixture was incubated another 30 minutes to repair the 5'-end single stranded overhang. The reaction was stopped by heat inactivation of the enzyme at 68°C for 10 minutes. DNA was phenol extracted once, ethanol precipitated and resuspended in TE.

EXAMPLE 2

CLONING OF RANDOM SHEARED DNA FRAGMENTS

The sonicated blunt end repaired HTLV-III DNA fragments were ligated into the SmaI site of the ORF expression vector pMR100 and transformed into host cell LG90 using standard transformation procedures. B-galactosidase positive phenotype of the transformant were identified by plating the transformed cell on ampicillin (25 ug/ml) containing McConkey agar plates and scoring the phenotype after 20 hours at 37°C.

EXAMPLE 3

HYBRID PROTEIN ANALYSIS

Ten milliliter samples of cells from an overnight saturated culture grown in L broth containing ampicillin (25 ug/ml) were centrifuged, the cell pellet was resuspended in 500 ul of 1.2 fold concentrated Laemmli sample buffer. The cells were resuspended by vortexing and boiling for 3 minutes at 100°C. The lysate was then repeated by being forced through a 22 gauge needle to reduce the lysate viscosity. Approximately 10 ul of the protein samples were electrophoresed in 7.5% SDS-PAGE (SDS-polyacrylamide) gels.

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Electrophoretic transfer of proteins from SDS-PAGE gels to nitrocellulose paper was carried out according to Towbin et. al.. After the transfer, the filter was incubated at 37°C for two hours in a solution of 5% (w/v) nonfat milk in PBS containing 0.1% antifoam A and 0.0001% merthiolate to saturate all available protein binding sites. Reactions with AIDS antisera were carried out in the same milk buffer containing 1% AIDS patient antisera that had been preabsorbed with E. coli lysate. Reactions were performed in a sealed plastic bag at 4°C for 18-24 hours on a rotatory shaker. Following this incubation, the filter was washed three times for 20 minutes each at room temperature in a solution containing 0.5% deoxycholic, 0.1 M NaCl, 0.5% triton X-100, 10 mM phosphate buffer pH 7.5 and 0.1 mM PMSF.

To visualize antigen-antibody interactions, the nitrocellulose was then incubated with the second goat antihuman antibody that had been iodinated with ¹²⁵I. The reaction with the iodinated antibody was carried out at room temperature for 30 minutes in the same milk buffer as was used for the first antibody. The nitrocellulose was then washed as previously described and exposed at -70°C using Kodak XAR5 film with an intensifying screen.

EXAMPLE 4

SCREENING OF THE HTLV-III ORF LIBRARY

BY COLONY HYBRIDIZATION

E. coli LG90 transformants were screened with HTLV-III DNA probes containing the DNA regions of interest (e.g. HTLV-III gag, env or Px gene specific

sequences). Colonies were grown on nitrocellulose filter and screened according to the procedure of Grunstein and Hogness by using a nick-translated HTLV-III DNA as hybridization probe.

The DNA fragment was in general exercise by restriction endonuclease digestion, gel purified, and ^{32}P -labeled to a specific activity of 0.5×10^8 cpm/ug by nick-translation (Rigby, P.W.J. et al., J. Mol. Biol. 113, 237 (1977)). Duplicate nitrocellulose filters with DNA fixed to them were prehybridized with 6xSSC (0.9 M NaCl/0.09 M sodium citrate, pH 7.0), 5X Denhardt's solution (Denhardt's solution: 0.02% each of polyvinylpyrrolidone, Ficoll and bovine serum albumin) 10 ug of denatured sonicated E. coli DNA per ml at 55°C for 3-5 hours. The filters were then placed in a fresh sample of the same solution to which the denatured hybridization probe had been added. Hybridization was permitted to take place at 68°C for 16 hours. The filters were washed repeatedly in 0.3XSSC at 55°C, and then exposed to x-ray film.

Industrial Applicability

This invention has industrial applicability in screening for the presence of HTLV-III DNA in body fluids and the diagnosis of AIDS.

Equivalents

Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are considered to be within the

scope of this invention and are covered by the following claims.